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GRANT NUMBER DAMD17-94-J-4351

TITLE: Isolation of a Receptor for WNT/Wingless Growth Factors

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REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (1 Sep 97 - 31 Aug 98)

4. TITLE AND SUBTITLE

Isolation of a Receptor for WNT/Wingless Growth Factors

5. FUNDING NUMBERS

DAMD17-94-J-4351

6. AUTHOR(S)

Nusse, Roel, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Stanford University
Stanford, California 94305

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The work done in this grant aims at elucidating the function of the *Wnt* signaling molecules in cancer. *Wnt* proteins are secreted and play important growth controlling roles, in particular in the mammary gland. They can act as oncogenes in mouse mammary tumors. The work was specifically aimed at identifying a receptor for *Wnt* proteins. Using cell biological and biochemical approaches we succeeded in finding a receptor for *wingless* called *frizzled*. During the past year, we have further characterized this receptor, as well as other genes in *Drosophila* involved in *wingless* function. We have also isolated a novel gene in *Drosophila* that modifies *wingless* signaling. This gene is highly conserved and may be involved in Wnt regulation in vertebrates as well.

14. SUBJECT TERMS

Breast Cancer, oncogenes, molecular biology of breast cancer, growth factors, receptors, signalling between cells, Drosophila, genetics

15. NUMBER OF PAGES 20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18 298-102

USAPPC V1.00

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Introduction

The goal of this grant, now at its end, has been to understand more about the function of the *Wnt* signaling molecules in cancer and in embryogenesis. The emphasis was on finding a receptor for *Wnt* proteins. *Wnt* genes encode secreted proteins involved in cell-to-cell signaling. The *Wnt* gene family includes a *Drosophila* gene *wingless*, which genetically has been well characterized. *Wnt* genes control growth, in particular in the mammary gland and, importantly, can act as oncogenes in mouse mammary tumors. Problems with working with *Wnt* proteins in vitro had precluded the isolation and characterization of *Wnt* receptors.
Our approach to identify a *Wnt* receptor was two-fold:

1. We used an assay for soluble *wingless* protein and an *in vitro* cell culture assay to identify *wingless* receptors.
2. In *Drosophila*, we performed genetic screens for modifiers of a *wingless* phenotype and clone those mutant genes.

Two years ago we found that the D*frizzled*-2 protein fulfills the criteria to act as a receptor for *wingless*. We thereby accomplished the major goal of this grant. During the past year, we have characterized the interaction between frizzled and wingless proteins further. In addition, we have identified and cloned a novel gene in Drosophila that modifies wingless signaling.

Body

1. Introduction, *wingless* signaling in vitro; identification of a receptor

Two years ago, we reported our finding son the identification of a receptor for the Wingless protein. We found that *Drosophila* S2 cells do not respond to the wg protein, indicating that they lack one or more components of Wg signaling. We tested whether transfection of receptor candidates would make S2 cells responsive to the *wingless* protein. One interesting receptor candidate was identified during the characterization of a large family of putative cell surface receptors with extensive homology to the *Drosophila* tissue polarity gene *frizzled* (*fz*). D*fz*2 is expressed in a *Drosophila* clone-8 cell line that is *wg*-responsive, but not in a non-responding S2 cells (the assay for *wg* activity being the stabilization and subsequent accumulation of the Arm; Van Leeuwen et al., 1994). After transfection with the D*fz*2 gene, S2 cells are able to transduce the *wg* signal. In addition, the S2 cells can now bind *wg* protein on their cell surface (Bhanot et al., 1996).

Hence, we have shown that the *Dfz-2* gene fulfills two criteria to be a receptor for the Wg protein: Wg binds to the *Dfz-2* and binding leads to a biological response; an increase in intracellular Arm concentration. In most vertebrates, more than 10 *Wnt* genes have been identified. As expected, there exists indeed a large family of fz-like genes in vertebrates, likely candidates for receptors for the other *Wnt* proteins (Wang et al., 1996). At this moment, there is no genetic evidence that *Dfz-2* is required for Wg signaling, as no mutants at the gene are available (reviewed in (Cadigan and Nusse, 1997). Evidence in our lab (Cadigan et al., 1998) in others have actually supported the view that Dfz2 is specific for wingless signaling (Zhang and Carthew, 1998).

Work done in year 4: Inhibiting wingless signaling by a dominant negative form of the receptor.

To examine whether Dfz2 functions as a receptor for Wingless in vivo, we created transgenic flies containing UAS binding sites for yeast Gal4 (Brand and

Perrimon, 1993) in front of a truncated Dfz2 cDNA predicted to encode the extracellular domain anchored to the cell surface via a glycerol-phosphatidyl inositol linkage. This truncated protein (GPI-Dfz2) binds wg protein in cell culture (Bhanot et al. 1996) but should not be able to transduce the signal to intracellular targets, since it lacks the seven transmembrane and intracellular domains. Therefore, if Dfz2 and wg can interact in vivo, GPI-Dfz2 should block wg signaling by acting as a sponge, binding the protein non-productively.

The Gal4 binding sites in the GPI-Dfz2 transgene allow it to be misexpressed in a variety of patterns through the use of Gal4 expressing lines (Brand and Perrimon, 1993). Expression of GPI-Dfz2 in the wing pouch of wing discs abolishes the expression of the wg targets ac and Dll. Experiments in the embryo, wing and eye all indicate that GPI-Dfz2 efficiently blocks wg signaling in these tissues (Cadigan et al., 1998). While these data do not conclusively demonstrate that the Dfz2 locus is required for wg signaling, they are consistent with that hypothesis and experiments described below strengthen this view.

Wg induces several bristle types at or adjacent to the margin and loss of Wg signaling in clones results in a lack of these bristles and notches in the wing blade. A similar notched phenotype is observed in animals containing 71BGal4, which is active in the wing blade primordium and a weak UAS-GPI-Dfz2 transgene. With a strong UAS-GPI-Dfz2 line, up to one third of the wing blade is missing. Thus, in addition to previously observed block in wg target gene expression in wing discs (Cadigan et al., 1998), GPI-Dfz2 does result in penetrant defects in the adult wing consistent with blocking Wg action.

To strengthen this connection between GPI-Dfz2 and Wg signaling, we examined whether mutations in *wg* or pathway components could modify the wing phenotype. Because of the cold sensitivity of Gal4, these animals showed only occasion notches. However, animals at 25°C always displayed notches when heterozygous for wg^{IN}. Similar genetic interactions were also observed with another *wg* allele (wg^{CX4}) and alleles of *dsh*, *porc* and *arm* ,which are required for Wg signaling (reviewed in Cadigan and Nusse, 1997). Thus GPI-Dfz2 acts antagonistically with Wg signaling at the genetic level.

If GPI-Dfz2 blocks Wg signaling by competing with the endogenous receptor for Wg binding, then it should have no effect on phenotypes generated by signaling components downstream of wg. Activation of the pathway in an *wg*-independent manner has been achieved by overexpression of *dishelved* (*dsh*; Cadigan and Nusse, 1996). In the eye, expression of *wg* and *dsh* using glass (GMR)-Gal4 resulted in a much smaller, glassy eye phenotype. The smaller eye is not due to a direct affect on morphogenetic furrow progression; not surprising since GMR-Gal4 is only active behind the advancing furrow. Co-expression of GPI-Dfz2 almost completely blocks the GMR-Gal4/UAS-wg phenotype but has only a subtle affect on UAS-dsh. **Therefore, GPI-Dfz2 blocks Wg signaling downstream of *wg* and upstream of *dsh* and *arm*.**

Work done in year 4: Loss of *fz* activity does not modify GPI-Dfz2 phenotypes in the wing and embryo

Like Dfz2, Fz can act as a Wg receptor in cell culture (Bhanot et al., 1996) and the extracellular domain can block Wg signaling (Zhang and Carthew, 1998). Unlike *Dfz2*, mutants for *fz* are available, though they do not suggest an obvious role in transducing the Wg signal. While *wg* is required for many essential developmental decisions) and mutants are embryonic lethal flies completely lacking *fz* activity survive to adulthood, displaying a planar polarity phenotype. One possibility is that *fz* may be redundant with other Frizzleds, like *Dfz2*. If this is the case, it may be possible to uncover a *fz* requirement in Wg signaling by looking in a sensitized background where Wg is limiting. Under these conditions, the removal of *fz* activity may reduce the levels of Wg signaling, altering the phenotype.

We have used the highly penetrant *wg*-like phenotypes generated by GPI-Dfz2 expression to test for genetic interactions with *fz*. As outlined above, 71B-Gal4/UAS-GPI-Dfz2 wing phenotypes are enhanced in a *wg* heterozygous background. However, no difference was observed in the degree of GPI-Dfz2 induced notching when both copies of *fz* were mutated. With levels of GPI-Dfz2 expression where no notching occurred or with severe notching, lack of *fz* activity also had no detectable effect on the phenotype.

Work done in year 4: wg signaling inhibits Dfz2 expression

To determine the expression pattern of Dfz2 in the wing pouch, whole mount in situ hybridization was performed to detect Dfz2 transcripts. Dfz2 levels are lowest at the D/V boundary, where wg protein is highest. wg signaling is responsible for the low Dfz2 mRNA close to the wg stripe, since Dfz2 expression is derepressed in wg^{ts} discs where wg activity is blocked for 24 hours prior to staining. To extend these findings we took advantage of UAS constructs expressing deleted versions of two wg signaling components, armadillo and dTCF, which constitutively activate or inhibit wg signaling, respectively (van de Wetering et al., 1997). Expression of activated arm throughout the wing pouch represses Dfz2 expression while expression of dominant negative dTCF in a Patched (Ptc) pattern (a stripe which runs perpendicular to the D/V wg stripe at the anterior/ posterior boundary) leads to derepression of Dfz2 in the Ptc domain. Thus wg signaling is responsible for the graded expression of Dfz2 in the wing pouch.

Work done in year 4: misexpression of Dfz2 expands wg target gene expression

To test whether the gradient of Dfz2 expression is important for normal wing development, we created transgenic UAS-Dfz2 flies to allow misexpression of Dfz2. All surviving animals have ectopic bristles on their wing similar to ectopic wg expression. Consistent with the hairy wing phenotype, IJ3 Gal4/UAS-Dfz2 discs have a dramatic increase in cells expressing high levels of ac and these cells are found at a greater distance from the wg stripe than in controls and presumably cause the ectopic bristles seen in adult wings. Thus, misexpression of Dfz2 at high levels throughout the wing pouch expands the domains of both short and long range wg targets (Zecca et al., 1996).

The increased activation of wg targets by misexpression of Dfz2 could be due to a heightened response of the cells to the wg signal, or a constitutive activation of the signaling pathway. To address this, we examined the effect of Dfz2 misexpression in wg^{ts} discs where wg activity was blocked. Both ac and Dll expression was dramatically reduced under these conditions, to levels seen in wg^{ts} discs under the

same conditions in an otherwise wild type background. This indicates that the primary effect of Dfz2 misexpression is to potentiate the ability of wg to signal to target cells.

Misexpression of Dfz2 or GPI-Dfz2 causes a dramatic post-transcriptional spread of wg protein (Zecca et al., 1996), with IJ3 Gal4/UAS-Dfz2 discs having high levels of wg several cells away from the RNA stripe. The ectopic wg protein is found on what appears to be the surface of the cells, and in contrast to endogenous wg, predominately basal laterally.

In conclusion, expression of the extracellular domain of Dfz2 efficiently blocks wg signaling (Cadigan et al. 1998), suggesting binding in vivo. In constrast to *Dfz2*, misexpression of *frizzled* in the developing wing has no affect on Wg signaling or Wg protein distribution. Conversely, misexpression of *fz* severely disrupted planar polarity in the wing and eye but *Dfz2* did not. Similar results have also been recently reported by Zhang and Carthew (1998). These data argue for a model where Dfz2 and Fz have distinct signaling activities, the former for Wg signaling and the latter for planar polarity.

Genetic analysis of Wg and planar polarity signaling is also consistent with a distinct signaling mechanism. The Wg signaling component Dsh is necessary for Fz planar polarity signaling but there is now abundant evidence that with this one exception, Wg and planar polarity pathways are non-overlapping (Boutros et al., 1998; Axelrod et al., 1998). Dsh appears to act in both pathways through different domains (Boutros et al., 1998; Axelrod et al., 1998). How Dfz2 and Fz interact with differently with Dsh to transduce either a Wg or planar polarity signal is now an important unanswered question in the field.

These data support the view that Dfz2 is a specific receptor for wingless in vivo. Together with our earlier data, we have therefore achieved much of our working goals of this grant.

2. A genetic screen for suppressors of a *wingless* phenotype in *Drosophila*

In our initial proposal, we proposed to take a second route to the identification of components of *wingless* signal transduction in *Drosophila*, by taking advantage of the genetic tools developed in this organism. By performing genetic screens for suppressors of a *wingless*-caused phenotype in the fly, one can uncover mutations in genes that are essential to generate this phenotype. Those genes could encode components of the *wingless* signaling pathway, including the receptor. An efficient way of identifying additional genes in a pathway is to perform a screen for dominant modifiers of a given phenotype (Brunner et al., 1997). To apply this approach to Wg signalling, an adult *wg* phenotype is required. **We reported initially** that Wg, expressed ectopically in the pupal eye disc via the sevenless promoter, specifically blocks the formation of interommatidial bristles (Cadigan and Nusse, 1996). No other detectable abnormalities were found in either pupal or adult transgenic eyes. Wg was shown to act at the level of the proneural genes to block the formation of the sensory organ precursor cells that give rise to the interommatidial bristles.

Over the past four years, we have continually performed a genetic screen that allowed not only for the identification of dominant suppressors on the autosomes that modified the adult Wg phenotype in the eye, but also allowed for the identification of dominant enhancers. Instead of the Wg cDNA, the temperature sensitive allele of *wg* (wg^{IL114}) (van den Heuvel et al., 1993) was used driven by the sevenless promoter (P[sew-Wg^{IL114}]). Growth of these transgenic flies at the intermediate temperature resulted in a sensitized background in which only half the number of interommatidial bristles are lacking.

During the Fourth year of this grant we have specifically analyzed a dominant enhancer encoding a novel protein, Tartaruga (Trt), that represses the Wg signalling pathway. During the past year, we have molecularly cloned the tartaruga gene.

Transgenic eyes have a temperature sensitive reduction in the number of interommatidial bristles. At the permissive temperature of 22°C, interommatidial bristles are normally formed. At 16°C, the restrictive temperature, a strong reduction in the number of interommatidial bristles is observed. These transgenic flies were crossed to ethyl methane sulfonate (EMS) mutagenized males and the F1, which was grown at an intermediate temperature of 17.6°C, was screened for either an enhancement or a suppression of the amount of bristles in the eye. In this way, a lethal complementation group consisting of two dominant enhancers was identified on the third chromosome that we named *tartaruga* (*trt*). This complementation group was further analyzed for interactions with *wg* outside of the eye. Observation of flies heterozygous for the alleles did not reveal any interactions with *wg*. Therefore, mosaic analysis was performed allowing the identification of interactions with *wg* when both copies of *trt* are removed.

In order to find out whether *trt* is enhancing *wg* activity during embryogenesis as well, the embryonic cuticle phenotype (Bejsovec and Martinez Arias, 1991) was analyzed of germline clone embryos. Germline clone embryos are embryos that lack any Trt wildtype product. They are derived from female germline mosaics that are homozygous mutant for *trt*. In these germline mosaic clones the maternal contribution of the nurse cells to the oocyte consists only of mutant Trt RNA. Germline mosaics were made with the autosomal FLP-DFS technique as reported by (Chou and Perrimon, 1992). The cuticle phenotype of these germline clone embryos was entirely naked, with only occasional denticles, which is in agreement with the enhancing effect of *trt* on *wg* activity. This was the first evidence that *trt* is enhancing *wg* activity outside of the eye as well.

The naked cuticle phenotype was seen in 33% of the embryos. The remaining 67% did not have any cuticle and did not show to have undergone any development at all (result not shown). The germline mosaics that give rise to these embryos were made using the trt^{10A20} allele. Germline clone mosaics using trt^{7A5-}

^{B} did not result in the production of any eggs. This may be due to the requirement of *trt* in oogenesis or to the effect of another mutation on the trt^{7A5-B} chromosome.

In order to establish whether the En domain in these germline clone embryos was broadened, Wg and En antibody stainings were performed. In*trt* germline clone embryos, we indeed noticed an expansion of the En domain, reminiscent of that seen in HS-Wg embryos. In addition, there was also an expansion of the Wg domain. This is an indication that *trt* causes a de-repression of Wg expression itself. Wg and En expressing cells covered the whole parasegment. In older germline clone embryos, a large variety in Wg and En expression patterns was observed. Invariably, the broadening of the En domain was more prominent to the posterior of the embryo. This phenotype was not rescuable by the presence of paternal wildtype gene product. Both zygotic homozygous and zygotic heterozygous germline clone embryos revealed the same Wg and En expression patterns, indicating that this phenotype is strictly caused by the lack of maternal wildtype Trt RNA.

Trt derepresses Wg at the transcriptional level

The expression pattern of Wg in *trt* germline clone embryos indicated that *trt* may be de-repressing Wg itself. In order to test this hypothesis, mosaic clones of *trt* were generated in imaginal discs which were subsequently stained with a rabbit Wg antibody. These mosaic clones were generated with the FLP-FRT system reported by (Xu and Rubin, 1993). For this purpose, the trt^{10A20} allele was recombined on a FRT chromosome and crossed to flies containing the myc-FRT chromosome. Recombination on the FRT elements was induced in flies containing both FRT chromosomes by activation of the flipase (FLP) gene through heatshocking 24-48 hrs or 48-72 hrs after egg laying (AEL). In this way, cells were produced that were either homozygous mutant for *trt* or that contained two copies of the myc gene. Staining with a myc antibody allowed for the localization of the mosaic clone and for the localization of the corresponding twin spot. Mosaic clones of *trt* in wing and leg imaginal discs as well as in the haltere and antennal discs indeed showed high

ectopic expression of Wg protein. However, only clones that were localized close to the endogenous Wg domain exhibited this ectopic expression. The mosaic clones that are homozygous mutant for *trt* were slightly larger and rounder than the corresponding twin spots.

Mosaic *trt* clones in adult wildtype eyes, wings and legs

The finding that trt^{10A20} clones in eye discs do not show any ectopic Wg expression was remarkable, since the interaction of *wg* and *trt* was initially found in the eye. Mosaic clones of trt^{10A20} cells in the adult wildtype eye were also generated with the FLP-FRT system. In this experiment, the trt^{10A20} -FRT chromosome was recombined with the w^+-FRT chromosome at 24-48 hrs AEL giving rise to homozygous *trt* mutant cells that had lost the w^+ eye marker. A mosaic *trt* clone lacked bristles and caused an irregularity in ommatidial structure. Closer analysis revealed that the lack of interommatidial bristles was a cell autonomous effect and therefore not attributable to the de-repression of a diffusible protein such as Wg.

Mosaic *trt* clones were also analyzed in wings and legs. In order to be able to detect these clones the y^+ body color was used as marker. Recombination was allowed to take place at different times in development between the trt^{10A20}-FRT chromosome and the y^+-FRT chromosome. Clones of cells homozygous for trt^{10A20}had lost the y^+ marker and were yellow in color. Upon heatshocking 24-48 hrs AEL, wings were shown to be completely misfolded and blistered (not shown). Furthermore, tumor-like outgrowths were observed especially at the distal tip of the wing. These outgrowths consisted of cells homozygous for trt^{10A20}. Mosaic clones were also generated later in development, at 48-72 hrs AEL, resulting in smaller clones that were less disruptive and therefore easier to analyze. These clones consisted of clumps of cells that were growing between the wing blades and had a disorganized pattern. The tumor-like growths were observed only within the *trt*

mutant mosaic clones and were therefore cell autonomous. Furthermore, these tumor-like phenotypes were observed throughout the wing, including sites in which *trt* mutant clones were shown not to induce ectopic Wg expression. Both the mosaic analyses in the eye and in the wing revealed that in addition to being a de-repressor of Wg at the transcriptional level, *trt* is able to interact with the Wg signalling pathway as well.

The generation of mosaic clones at 24-48 hrs and at 48-72 hrs AEL did not result in flies with clones in legs. Heatshocking even later in development, namely at 72-96 hrs AEL, resulted in the eclosion of flies with distorted legs. These legs had a disorganized proximodistal axis, with fusions of leg segments, and an increased circumference. The *trt* mosaic clones were invariably shown to be smaller than the size of the affected leg, indicating a strong non-autonomous effect of the mutation (Struhl and Basler, 1993). A clone on the dorsal side of the tibia from a female first leg did not show any phenotype at all. However, a dorsal clone on the tarsal segments of the third leg showed fusion of these segments. A clone positioned at the ventral side of the tarsus of a female first leg showed an enlargement of the ventral tranverse bristle rows.

Cloning of the *trt* gene

Three lethal transposon insertion lines, P(01814), P(j4A5), and P(rN672) which were mapped to 67E-5-7 were found not to complement trt^{10A20}. The first two P lines did not complement $trt^{7A5\text{-}B}$ either, but P(rN672) did. Furthermore, these three P lines did not complement each other. In 5-10% of the embryos from these P stocks a naked cuticle was observed. In order to provide evidence that the transposons themselves are disrupting *trt* function the transposons were excised in the presence of the _2-3 recombinase. This resulted in flies that complemented trt^{10A20}, indicating that the transposons were indeed the cause of the disruption of the *trt* gene. Furthermore, this experiment led to a few imprecise excisions of the transposon elements, which resulted in the generation of four new alleles, j4A5-16.1. j4A5-18.1, rN672-85.1, and 01814-46.2.

The finding of these P lines was the starting point for the cloning of the *trt* gene. The plasmids of these P lines were rescued and the flanking genomic DNA was mapped by means of restriction enzyme analysis. The flanking genomic DNA was used as probes on Northern blots consisting of poly-A^+ RNA from an overnight collection of wildtype embryos. The flanking genomic DNA from P(j4A5) and that from P(rN672) detected the same transcripts on this Northern blot, namely a faint 6.3 kb and a stronger 4.7 kb band. The signals were only seen after a three day exposure suggesting a very low abundance of the transcript. The flanking genomic DNA that detected these transcripts was used to screen a cDNA library from a 0-5 hr embryo collection of wildtype embryos. In this way, a 3.5 kb cDNA was isolated , which appeared to be identical to Drosophila EST LD18074 and to include LD18441. This cDNA contained a 5' UTR of 706 nucleotides, an open reading frame (orf) of 2751 nucleotides and a 3' UTR of 180 nucleotides. The Trt cDNA showed similarities to human, mouse and C.elegans EST clones. Alignment of these EST clones to the Trt cDNA revealed a conserved region from nucleotide 444-570 in the Trt cDNA. Extensive search for known protein motifs within the Trt orf showed a small similarity to the C2H2 type of zinc finger proteins. The flanking genomic DNA was recovered by plasmid rescue and used to probe a northern blot of poly A+ RNA from an overnight collection of embryos. The genomic recovered from P(j4A5) and from P(rN672) both detected a faint 6.3 kb band and a stronger 4.7 kb band.

The flanking genomic DNA from P(j4A5) was then used to screen a 0-4 hour embryonic cDNA library, and a 3.5 kb cDNA clone was isolated. After sequencing 800 base pairs, we performed a BLAST search (reference) and found a Drosophila EST clone LD18074 which was identical in sequence and by restriction mapping. LD18074 was then fully sequenced and used for rescue experiments.

The open reading frame (ORF) encoded a 917 amino acid protein. In vitro transcription and translation of the cDNA produced a protein of approximately 100 kDa which corresponds to the predicted size of 96 kDa.

Searching the EST database, we found homologous human, mouse, and C. elegans EST clones. Alignment of the partial cDNA clones with the Drosophila

sequence shows a highly conserved region of ?% similarity. Within this region, there is a zinc finger motif which is similar to the zinc fingers of GATA transcription factors.

To proof that this cDNA is indeed encoding the Trt protein, transformant lines were generated that carry the UAS-LD18074 construct. Trt^{10A20}/TM6,Tb was crossed to$trt^{7A5\text{-}B}$/TM6,Tb in the presence of the presumptive Trt transgene which was driven by arm-GAL4 and grown at 18°C. As control, the same cross was performed in the absence of the *trt* transgene. In this way, rescue was shown until puparium formation. trt^{10A20}/ $trt^{7A5\text{-}B}$ is embryonic lethal except for a percentage of 3% of escapers that develop until puparial stage. By means of the Chi^2 test, however, we were able to repeatedly show that in the presence of the Trt transgene, a significantly higher amount of embryos hatch and develop until puparial stage.

This provides evidence that we indeed cloned the cDNA encoding the Trt protein, a novel gene interacting with the wingless pathway. We have therefore achieved another major goal of this grant.

Conclusions

Over the past year, we have made significant progress. The main goal of the project, the identification of a *wingless* receptor, had been accomplished before but we have added significant information on the function of this receptor in vivo. Further work will address the biochemical mechanism of signal transduction by the *Dfz2* receptor, and the interactions between other members of the *frizzled* receptor gene family and the various *Wnt* proteins. In addition, in year 4, we cloned a novel gene in Drosophila interacting with wingless signaling. This gene is highly conserved and mouse and human homologs have been isolated.

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